Arch Microbiol (1985) 141:266–268

Archives of
Microbiology

Metabolic effects of manganese deficiency in *Aspergillus niger*: evidence for increased protein degradation

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Abstract. The effect of manganese deficiency on macromolecule synthesis has been studied in a citric acid producing strain of *Aspergillus niger*: pulse labelling experiments showed that the synthesis of both protein and RNA was not influenced by the presence of manganese; however, increased protein degradation occurred under manganese deficiency. This was also reflected by the increased activity of an intracellular proteinase activity under these conditions. In replacement cultures addition of inhibitors of RNA, DNA or protein synthesis revealed that only emetine and cycloheximide (which both act at the ribosome) successfully antagonized the adverse effect of manganese ions on citric acid accumulation. Manganese deficiency was also characterized by a decreased portion of polysomes and 80 S ribosomes.

Key words: Manganese deficiency – *Aspergillus niger* – Citric acid accumulation – Protein degradation

Aspergillus niger is known to accumulate high quantities of citric acid in the medium when grown under certain defined conditions, among which manganese ion deficiency [Mn(–)] is a very important parameter (cf. Röhr et al. 1983). The role of this deficiency in citric acid overproduction has been investigated during the last years: Kubicek et al. (1979) observed elevated pool concentrations of certain amino acids and NH_4^+ under conditions of Mn(–). Habison et al. (1983) showed that these physiological concentrations of NH_4^+ (up to 15 mM) can relieve phosphofructokinase from feedback inhibition by citrate (intracellular concentration up to 4 mM). Since phosphofructokinase is the main metabolic control point in citric acid biosynthesis in *A. niger* (Röhr et al. 1983), manganese deficiency should be responsible for the apparent "deregulation" of glycolysis.

The molecular target of Mn(–), however, is still unclear: Kubicek et al. (1979) have supposed that the appearance of elevated concentrations of NH_4^+ and amino acids under Mn(–) is the result of an imbalance in protein or nucleic acid turnover. In the following communication we will present first evidence for an increased rate of protein degradation and some disarrangement in ribosome function under Mn(–) in *A. niger*.

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Materials and methods

1. Strain and culture conditions. Aspergillus niger B60 (Röhr et al. 1979) was used throughout these studies; its maintenance as well as cultivation under citric acid producing conditions have been reported previously (Kubicek and Röhr 1978).

2. Measurement of protein and RNA synthesis. Radioactive U-^{14}C-leucine (1 μCi) + 10 mg non-labelled leucine (or U-^{14}C-uridine, 10 μCi, + 10 mg non-labelled uridine) were added at predetermined intervals during the cultivation into fermentation flasks containing 20 ml of culture broth and incorporated into the cell for 15 min. At the end of cultivation 5 ml of 50% (w/v) trichloroacetic acid were then added to the flasks. The mycelia were then separated by filtration through sinter funnels, washed twice with 10% (w/v) cold trichloroacetic acid, washed further twice with acetone and dried in a desiccator. The radioactivity of 10 mg of dry mycelium was measured in 5 ml of scintillation solution in a Packard-TriCarb 300 C liquid scintillation system.

3. Determination of protein and RNA degradation. U-^{14}C-leucine (10 μCi) + 100 mg non-labelled leucine (or U-^{14}C-uridine, 10 μCi, + 100 mg non-labelled uridine) were added to 20 ml of culture broth and cultivated further for 48 h. Then the contents of 3–6 flasks were pooled, the mycelia centrifuged, washed with physiological saline and aliquots were distributed into the same number of flasks containing 20 ml of the filtrate of a culture (unlabelled) of the same age, additionally containing 100 mg of unlabelled leucine (or uridine) and cultivated for further 30 min. The experiments were terminated by addition of trichloroacetic acid as described above. All subsequent steps were carried out in essentially the same way. The radioactivity of the mycelium at the time of pooling the flasks served as a control.

4. Miscellaneous techniques. Intracellular protein was extracted with 0.1 N NaOH as described previously (Kubicek et al. 1979) and quantified by the method of Bradford (1976). Molecular sieve chromatography was carried out on a Biogel P-200 column (25 × 280 mm) previously equilibrated with 0.1 M phosphate buffer pH 6.75 containing 1 mM EDTA and 0.1 mM 2-mercaptoethanol. For determination of the portion of active ribosomes in protein synthesis, the method of Orlowski and Sypherd (1978) was used. All other analytical techniques were essentially as described previously (Kubicek et al. 1979).

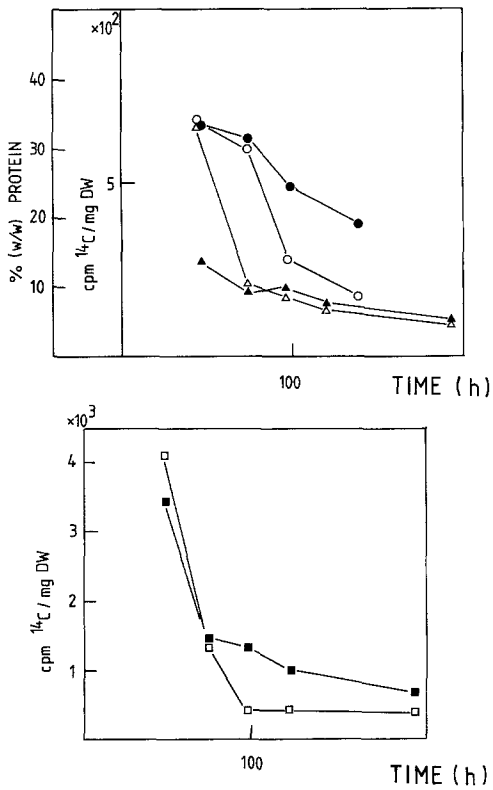

Fig. 1. Rate of protein accumulation (●, ○), protein synthesis (▲, △) and protein degradation (■, □) during citric acid accumulation in the presence (full symbols) or absence (empty symbols) of 5×10^{-5} M Mn^{2+}. Protein synthesis is indicated by the incorporation of ^{14}C label during 15 min, protein degradation is indicated by the ^{14}C label remaining after 30 min dilution of incorporated ^{14}C-leucine

Results and discussion

As we have previously shown (Kubicek et al. 1979), the intracellular protein pool in *Aspergillus niger* reaches a maximum during the rapid phase of growth, which is independent of the presence or absence of manganese in the culture medium. In contrast during the idiophase, the decrease in the amount of intracellular protein is clearly more pronounced in mycelia grown under Mn(−) conditions. By means of pulse labelling with ^{14}C-leucine it was now shown that the biosynthesis of protein occurs at the same rate both under manganese deficiency as well as manganese addition (5×10^{-5} M). However, when mycelia which have been previously fed with labelled leucine were replaced in a medium containing unlabelled leucine, the radioactivity of Mn(−) grown mycelia dropped of more rapidly than that of Mn(+) grown mycelia, indicating a more rapid degradation of the protein pool under manganese deficiency (Fig. 1).

By gel filtration of the intracellular proteins on Biogel P-200 it was shown that during the idiophase in Mn(−) mycelia the protein pool comprises a rather higher portion of low molecular weight (<M 20,000) polypeptides. With regards to the pulse labelling studies reported above, we consider them to be most likely protein degradation products. SDS-PAGE (Laemmli 1970) provided evidence that rather the complete protein pool than certain individual proteins were affected by the degradation.

Table 1. Proteinase activity during citric acid fermentation under manganese deficient [Mn(−)] and manganese containing [5×10^{-5} M Mn^{2+}, Mn(+)] conditions[a]

h of cultivation	Mn(+)	Mn(−)
48	0.026	0.018
96	0.037	0.109
192	0.045	0.208

[a] Values are given as increases in absorbancy at 366 nm per mg of intracellular protein. The pH for activity determination was 4.6. Proteinase was extracted from the mycelium as described by Habison et al. (1983) using 0.1 M phosphate buffer pH 7.0 containing 5 mM EDTA and 5 mM 2-mercaptoethanol

The increased protein degradation rate observed is most likely due to the activity of an elevated activity of a proteinase: Mn(−) conditions are characterized by a significantly increased proteinase activity (Table 1). Although the optimal pH of this proteinase is 4–5, approximately 30% of activity is still observed at pH 7.0; the enzyme should thus be active at a presumably intracellular pH value, and may well be responsible for the observed protein degradation.

Pulse labelling experiments with ^{14}C-uridine showed no significant differences in protein synthesis or degradation between Mn(−) and Man(+) mycelia; we thus conclude that manganese deficiency does not affect RNA turnover.

Since the increased protein degradation indicated above may be the consequence of manyfold reasons, we have further used inhibitor experiments in the attempt to compare the "in vivo" activity of selected steps in protein or nucleic acid synthesis during Mn(+) or Mn(−) cultivation. The results thereby obtained are documented in Table 2: as can be seen, Mn(−) as well as Mn(+) mycelia were both sensitive towards most of the inhibitors with regard to growth as well as citric acid production, although the degree of sensitivity was not identical. However, most striking is the stimulation of citric acid accumulation by cycloheximide and (to a lesser extent) by emetine under Mn(+) but not Mn(−) conditions. Since both antibiotics attack at the ribosomal elongation step, the results indicate that some disfunction at this point might be at least one of the targets of manganese deficiency. When manganese ions were fed to a Mn(−) grown culture of *A. niger* with concomitant addition of cycloheximide a direct relationship was found between the concentration of Mn^{2+} added and the amount of cycloheximide required to suppress manganese action. Such an antagonism was not observed with 5-fluorouracil, actinomycin C or mitomycin.

The effect of Mn(−) on ribosomal protein synthesis was also seen from sucrose gradient centrifugation analysis of *A. niger* ribosomes: Mn(−) conditions were characterized by a decrease in the proportion of polyribosomes and 80 S ribosomes and a reciprocal increase in the monosome portion within the ribosome population. This was not due to increased RNAse activity under Mn(−) conditions, since essentially the same results were obtained in the presence of diethylpyrocarbonate which inhibits RNAse activity. Since the ratio of polysomes to monosomes has been interpreted as an indicator of functional protein synthesis (Orlowski and Sypherd 1978), these results coincide with the results from the inhibitor experiments.

In summary, we have supplied preliminary evidence for our previous hypothesis (Kubicek et al. 1979) that Mn(−)

Table 2. Influence of metabolic inhibitors on citric acid accumulation and its susceptibility to Mn^{2+} ions[a]

Inhibitor		Citric acid/dry weight			
		–	50 ppb Mn^{2+}	100 ppb Mn^{2+}	200 ppb Mn^{2+}
–		100	72.3	61.6	56.3
Cycloheximide	20 µg/ml	83.2	84.2	78.2	73.1
Chloramphenicol	50 µg/ml	121.3	73.0	59.8	52.4
5-Fluorouracil	10^{-4} M	21.1	19.0	47.8	19.0
	10^{-5} M	56.3	35.9	30.3	33.1
6-azauridine[b]	100 µg/ml	44.4	–	51.4	50.6
		(49.6)	–	(57.1)	(39.9)
Emetine	30 µg/ml	127.2	73.4	66.7	70.4
Actinomycin	3 µg/ml	87.4	–	64.3	–
	10 µg/ml	78.8	–	59.1	–
Rifampicin	100 µg/ml	100	–	57.2	–
Mitomycin	100 µg/ml	38.4	–	23.9	–

[a] Mycelia were pregrown in Mn(–) for 50 h and then supplemented with the amount of Mn^{2+} ions and inhibitor as indicated. After further 36 h of cultivation, the flasks were analyzed for critic acid and dry weight. All values given are expressed as the ratio of the increase in extracellular citric acid to the increase in biomass dry weight during these 36 h, indicated as percents of the value obtained for a citric acid fermentation in the absence of Mn^{2+} ions and inhibitors. Values are means of at least 3 separate determinations

[b] 6-Azauridine was not taken up by the hyphae unless amphotericine B (10 µg/ml) was also added. The values in brackets indicate the effect of amphotericine B alone

growth conditions lead to an impaired protein turnover, which is caused by an increased rate of protein degradation. Concomitantly a disfunction in ribosomal activity is also apparent. Although we have carried out a number of additional experiments we are at present still unable to explain the possible link between these two Mn(–) dependent phenomena (Ma 1982). However, it seems possible that some ribosomal disfunction could lead to the synthesis of "false" proteins which themselves are substrates of increased protein degradation.

Acknowledgements. The authors thank Prof. K. Buchtela, Institute for Radiochemistry, TU Wien, G. Wiche, Institute of Biochemistry, University of Vienna, and H. Redl, Institute for Traumatology, Vienna, for making available laboratory facilities during the course of these studies. H. Ma was a recipient of a grant by the Peoples Republic of China. Thanks are also due to Prof. H. Kinzel for his support and encouragement during the course of this work.

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Received January 7, 1985/Accepted February 21, 1985